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Plant species diversity affects soil-atmosphere fluxes of methane and nitrous oxide

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Author contributions: PAN and RLB conceived and designed the present study with input from MSL and NB. Trace gas flux measurements and soil analyses were carried out by PAN, MSL and RLB. XLR and FP analyzed functional gene abundances. PAN analyzed the data with input from XLR and RLB. PAN wrote the manuscript with input from all co-authors.

Abstract

Plant diversity effects on ecosystem functioning can potentially interact with global climate by altering fluxes of the radiatively active trace gases nitrous oxide (N_2O) and methane (CH_4). We studied effects of grassland species richness (1 to 16) in combination with NPK-fertilizer application (N:P:K = 100:43.6:83 kg ha^{-1} a^{-1}) on N_2O and CH_4 fluxes in a long-term field experiment. Soil N_2O emissions, measured over two years using static chambers, decreased with species richness unless fertilizer was added. N_2O emissions increased with fertilization and the fraction of legumes in plant communities. Soil CH_4 uptake, a process driven by methanotrophic bacteria, decreased with plant species numbers, irrespective of fertilization. Using structural equation models, trace gas fluxes were related to soil moisture, soil inorganic N concentrations, nitrifying (NEA) and denitrifying enzyme activity (DEA), and the abundance of ammonia oxidizers, nitrite oxidizers and denitrifiers (quantified by real-time PCR of gene fragments amplified from soil DNA). These analyses indicated that plant species richness increased soil moisture, which in turn increased N cycling-related activities. Enhanced N cycling increased N_2O emission and soil CH_4 uptake, with the latter possibly caused by removal of inhibitory NH_4^+ by nitrification. The moisture-related indirect effects were surpassed by direct, moisture-independent effects opposite in direction. Microbial gene abundances responded positively to fertilizer but not to plant species richness. The response patterns we found were statistically robust and highlight the potential of plant biodiversity to interact with climatic change through mechanisms unrelated to carbon (C) storage and associated CO_2 removal.

Keywords: Functional genes, Jena experiment, microbial activities, nitrification and denitrification, structural equation modelling.

Introduction

Recent syntheses of experimental research have shown that effects of plant diversity loss on ecosystem functioning can be of similar magnitude as those of other global changes, including warming and drought (Hooper et al. 2012). However, effects of plant diversity interact with other global changes, with mechanisms operating at spatial scales ranging from the plot scale to the globe. For example, global change drivers may affect ecosystem functions directly and indirectly via alterations of plant diversity (Balvanera et al. 2006). Plant diversity changes can alter ecosystem carbon (C) and nutrient cycling, thereby changing the ecosystem-atmosphere exchange rates of greenhouse gases and thus affect climatic drivers at the global scale.

Carbon dioxide (CO_2) is the dominant anthropogenic greenhouse gas, but nitrous oxide (N_2O) and methane (CH_4) also are important drivers of anthropogenic climate change. Despite their low atmospheric volume mixing ratios, they account for over 25% of the radiative forcing increase since pre-industrial times because of their high warming potential relative to CO_2 (IPCC 2007). To date, most experimental plant diversity studies have focused on ecosystem functions related to plant primary productivity and the ecosystem's C cycle (e.g., biomass, photosynthesis, leaf area, litter decomposition), which are processes directly relevant for the land-atmosphere exchange of CO_2. However, plant diversity effects on land-atmosphere fluxes of N_2O and CH_4 have only been studied in few mesocosm studies (N_2O: Abalos et al. 2014; Chang et al. 2014; Sun et al. 2013; N_2O and CH_4: Niklaus et al. 2006), and relatively little is yet known on responses of the underlying nitrogen (N) cycling processes to plant diversity (Le Roux et al. 2013).

N_2O is released from soils as an intermediate or by-product of microbial N cycling (cf. Cabello et al. 2009 for a comprehensive overview of soil N transformations). Under oxic conditions, some N_2O produced as a by-product of nitrification will escape to the atmosphere. Under more anoxic conditions, nitrate is sequentially reduced to N_2O, which generally is lost from soils if it is not further reduced to molecular nitrogen (Firestone and Davidson 1989). Soil N_2O emissions are controlled by many factors (Robertson and Tiedje 1987) including the availability of inorganic N, in particular ammonium and nitrate. Soil moisture also is important because it affects gas diffusivity and oxygen supply. At low water filled pore space, oxygen is readily available and soil organic matter is mineralized, yielding NH_4^+ and promoting nitrification and associated N_2O emissions. At high water filled pore space, nitrification ceases and nitrate is consumed by denitrification, which is the dominant source of N_2O emissions from soils. At even higher water filled pore space, N_2O emissions drop because most of the N_2O produced is reduced to molecular nitrogen.

Soils can be both sources and sinks of atmospheric CH_4. On an annual basis, most aerobic soils are a net sink for atmospheric CH_4, although periodic emissions can occur when soils are wet. This soil CH_4 sink is essentially driven by the consumption of CH_4 by methanotrophic bacteria, which use CH_4 as a source of carbon and energy (Dunfield 2007). At very low redox potential (typically found in wetlands), however, methanogenic archaea produce CH_4 through several pathways, consuming organic substrates or CO_2 (Conrad 1996). Non-microbial soil CH_4 emissions have recently been described (Wang et al. 2013), but the quantitative importance of this process in natural ecosystems remains to be evaluated. Methanogens and methanotrophs often co-occur in soils, with methanotrophs acting as a biofilter that oxidises CH_4 produced by methanogens in anoxic soil domains. On the other hand, occasional methanogenesis can prime methanotrophic activity, leading to increased uptake of atmospheric CH_4 (West and Schmidt 2002). Several links have been established between CH_4 oxidation and N transformations, with complex, positive and negative correlations between the two (Bodelier and Laanbroek 2004). Ammonia has been shown to inhibit CH_4 oxidation, at least in laboratory cultures (Hanson and Hanson 1996), but on the other hand inorganic N is an essential nutrient for most methanotrophs. Further, differential stratification of these processes may occur in the soil, with effects that may be restricted to some soil layers and therefore not necessarily generate effects on soil-atmosphere CH_4 fluxes (Stiehl-Braun et al. 2011a).

While the fundamental processes involved in soil N_2O and CH_4 transformations are reasonably well understood, the ecology of the involved organisms in complex field ecosystems is far from being as clear (cf. Bodelier and Laanbroek 2004; Robertson 1989). Predicting N_2O and CH_4 fluxes is difficult given the large number of factors and organisms involved, which interact in ways difficult to predict and change in space and time. Nevertheless, we argue that plant diversity has the potential to alter trace gas fluxes via a range of mechanisms that can reasonably be predicted and tested. First, more species-rich plant communities often lead to tighter N cycling, with less inorganic N accumulating (Ewel et al. 1991; Niklaus et al. 2001; Tilman et al. 1996). This could affect nitrification and denitrification and associated N_2O emissions, and possibly also CH_4 dynamics. Second, increased productivity at higher plant diversity may also translate into an improved supply of organic substrates for soil microbial communities, which could increase heterotrophic activity, lead to higher oxygen consumption and increase the amount of soil volume with a redox potential sufficiently low for denitrification (Sexstone et al. 1985). Third, water use may increase with plant diversity, resulting in reduced soil moisture and increased soil diffusivity (Caldeira et al. 2001; Leimer et al. 2014a; Spehn et al. 2000), which may affect both N transformations and soil CH_4 uptake (Ball et al. 1997). Fourth, altered root densities and architectures may lead to changes in soil aeration, spatio-temporal organic C deposition or N use patterns. Fifth, atmospheric N_2 fixation by legumes will have particularly strong impacts

on N cycling, and has been identified as strong determinant of ecosystem responses in many plant diversity experiments (Oelmann et al. 2007; Spehn et al. 2002). With respect to CH_4, the study by Niklaus et al. (2006) hints at the complexity of interactions at play, with plant diversity effects on CH_4 consumption changing from positive to negative when a soil disturbance treatment was applied in the field.

Here, we present the first study investigating the effects of plant species diversity and fertilization, together with their interaction, on fluxes of N_2O and CH_4 under field conditions. We investigated the dynamics of these trace gases and the underlying mechanisms at several levels. First, we measured *in situ* flux rates of N_2O and CH_4; second, we assessed the enzymatic potential of key N transformations functionally linked to these fluxes; third, we quantified the abundance of nitrifiers and denitrifiers using quantitative PCR of selected functional and ribosomal genes. We further assessed soil environmental conditions (temperature, moisture) and concentrations of inorganic N species. We were interested whether higher plant species richness would lead to more complete N capture and therefore reduced nitrification and denitrification rates and associated N_2O emissions. We further aimed at testing whether plant diversity effects operated via changes in soil moisture, and whether there was evidence that nitrification promotes soil CH_4 uptake by removal of potentially inhibiting NH_4^+.

Materials and Methods

Study site and experimental design

We studied soil-atmosphere trace gas fluxes in a large grassland biodiversity experiment near Jena, Germany (50°55'N, 11°35'W, 130 m above sea level). In 2002, experimental grassland communities varying in diversity were established on an Eutric Fluvisol with a texture progressing from sandy loam to silty clay with distance from the adjacent Saale river. The present study focused on 78 plots (20 x 20 m) sown with 1, 2, 4, 8 or 16 herbaceous species (see Roscher et al. 2004 for a complete description of the experimental design). Plot community composition was determined by random selection of species from a 60-species pool, with the constraint that species richness and plant functional type richness were as orthogonal as possible. Plant species had previously been assigned to functional types (grasses, small non-legume herbs, tall non-legume herbs, and legumes) based on a cluster analysis combining a large number of morphological and functional traits (Roscher et al. 2004). Starting in 2005, factorial management treatments were established (Weigelt et al. 2009) in subplots (1.6 × 4 m) located along one side of the large plots. In the present study, we analyze data from the main plot ("control" subplots) and the subplot that was managed identically except that fertilizer pellets were added after each mowing ("fertilized" subplots), at a rate of 100kg N ha^{-1} a^{-1}, 43.6 kg P ha^{-1} a^{-1} and 83 kg K ha^{-1} a^{-1}. In total, the study thus presents a split plot design with 70 plots (unit of replication for plant species composition and thus plant species richness) and 2×70=140 subplots (unit of replication for fertilizer application).

Soil-atmosphere trace gas fluxes

We measured soil-atmosphere fluxes of N_2O and CH_4 on July 20 and October 17, 2007, and on March 11, April 29, and twice on June 8, 2008 (one day-time and one night-time measurement; Table 1), using static chambers that had been installed in all control and fertilized subplots that were part of this study. The static chambers were 32 cm in diameter and lowered 14 cm into the ground, leaving 11 cm extending above ground. During flux measurements, air-tight lids were fitted and headspace samples collected 5, 20 and 35 min after chamber closure. These samples were injected into pre-evacuated exetainers and analyzed for N_2O and CH_4 concentrations in the laboratory (Agilent 6890 gas chromatograph equipped with a flame ionization and an electron capture detector, Agilent Technologies Inc., Santa Clara, CA, USA). Concentrations were determined based on calibration against four standard gases with four different concentrations of CH_4 and N_2O (see Hartmann et al. 2011 for details of the experimental setup). Gas exchange rates were calculated by linear regression of headspace concentrations against time. Concentration changes were linear

with time, and regressions explained >95% of the variation in gas concentration (R^2>0.95), unless flux rates were very low.

Soil sampling

In October 2007 and again in June 2008, we collected eight 1.5 cm diameter × 15 cm depth soil cores per subplot within 50 cm of the respective static chamber used for N_2O and CH_4 flux measurements. Samples were pooled by subplot and sieved (2 mm mesh). A sub-sample was stored at –18 °C for molecular analyses, while another subsample was stored at 4°C for potential nitrifying and denitrifying activity analysis.

Nitrifying and denitrifying enzyme activity

Nitrifying (NEA) and denitrifying (DEA) enzyme activities were determined in laboratory assays, quantifying transformation rates of NH_4^+ and NO_3^- under standardized conditions, with neither water availability nor substrate concentration limiting. The goal was to quantify potential activity rates, eliminating limitations by substrate availability or environmental conditions that may have existed in the field.

NEA was measured at soil pH using the method described in Patra et al. (2005). NEA was determined as the difference between soil nitrate concentration before and after a 7-hour incubation of 10 g of soil under conditions favoring nitrification (28°C, addition of 0.2 mg $(NH_4)_2SO_4$-N g^{-1} dry soil, soil moisture equivalent to 70% water holding capacity).

DEA was assessed over a short time by making all the factors affecting denitrification rate non-limiting (Smith and Tiedje 1979; Tiedje et al. 1989). DEA was determined as the linear production rate of N_2O, measured by gas chromatography (Agilent P200, Santa Clara, CA) over an 8-hour incubation of 10 g soil under conditions favouring denitrification (28°C; 90:10 He-C_2H_2 atmosphere providing anaerobic conditions and inhibition of N_2O-reductase activity; addition of 1 mg glucose-C, 1 mg glutamic acid-C and 0.1 mg NO_3^--N per gram dry soil; soil moisture content equivalent to 100% water holding capacity).

Nitrifier and denitrifier abundances

Soil DNA was extracted with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). The abundance of beta-proteobacterial ammonia oxidizers, i.e. known ammonia oxidizing bacteria (AOB) in soil, was measured by quantitative PCR (qPCR) according to Le Roux et al. (2008), targeting 16S rRNA gene sequences that are specific for this group (Hermansson and Lindgren 2001). $5.72 \cdot 10^8$ to $5.72 \cdot 10^2$ copies of standard DNA (purified AOB 16S rRNA gene PCR product of ATCC19718 *Nitrosomonas europaea*) were used.

The abundance of *nxrA* gene copies from *Nitrobacter*-like nitrite oxidizing bacteria (NOB) was quantified according to Attard et al. (2010). Tenfold standard serial dilutions

ranging from 10^{7} to 10^{1} *nxrA* copies of genomic DNA from *Nitrobacter hamburgensis* X14 (DSMZ 10229) were used.

Denitrifier abundance was estimated by qPCR targeting fragments of the *nirK* and *nirS* genes encoding the copper and cd1 nitrite reductases, respectively. Quantitative PCR assays were carried out from soil DNA with Lightcycler 480 (Roche Diagnostics, Meylan, France). For *nirK*, thermal cycling and standard curve were carried out according to Henry et al. (2004), except that the last cycle with an annealing temperature of 58 °C was repeated 45 times. *nirS* fragments were amplified as described by Baudoin et al. (2009), using tenfold serial dilutions of genomic DNA of *Pseudomonas aeruginosa* PA14 as standards. We checked melting curves and amplified *nirK* or *nirS* fragment length after running an agarose gel of randomly chosen final PCR products.

For all quantitative PCR assays, measurements were performed in triplicate. Melting curve analysis confirmed the specificity of the amplifications, and possible inhibitory effects of co-extracted humic compounds in soil extracts were checked by dilution series, but no inhibition was observed.

Soil moisture and inorganic N concentrations

Soil moisture was determined gravimetrically in all fresh samples collected for molecular analysis. Soil ammonium and nitrate concentrations were determined colorimetrically (SAN+, Skalar, Netherlands) after extraction of fresh soil (20 g dry mass equivalent) with 60 mL of 2 M KCl (30 minutes) and filtration.

Data analysis

All data were analyzed by ANOVA based on mixed-effects models reflecting the experimental design (ASReml 3.0, VSN International, UK). The models included the main design variables plant species richness (as log-linear contrast) and fertilizer application, and their interaction. The specific composition of plant species communities was included as a random term to ensure adequate testing of the species richness effect (note that this random term is equivalent to "plot" since specific plant community compositions are not replicated at the plot level). The Jena Experiment is located on a flood plain adjacent to the Saale river, and spatial, treatment-independent, gradients in soil conditions across the site affected the processes we investigated. We therefore fitted the Cartesian coordinates X and Y indicating the plot's position, plus their 2^{nd} order terms (X·X, Y·Y, X·Y) to account for non-linearity in spatial gradients and for interactions between the two cardinal directions (i.e. allowing for a response surface that may be curved in a direction that does not align with the main coordinate axes). This spatial model explained more variance than fitting the design's standard blocks which were placed perpendicular to the river.

The different variables necessitated a range of transformations due to their specific distributions. In part, these distributions result from biological processes; however, they also are the result of specific measurement procedures such as the PCR reaction, which is an exponential process and results in a corresponding distribution of measurement errors. All gene abundances were therefore log-transformed. Soil CH_4 uptake rates as well as nitrification and denitrification enzyme activities were analyzed untransformed. Finally, soil N_2O oxide emissions resembled a log-normal distribution at large values but included very small and negative values due to measurement error, precluding log-transformation (which further would have over-emphasized differences between very small fluxes). We found that taking the fourth square root (with sign reconstruction for negative values), and shifting the abscissa of the first and third quadrant so that the two legs of the function merged with unity slope, resulted in a near-perfect normal distribution of residuals. However, analytical results did not change fundamentally when other transformations were chosen.

After testing for significances of effects of species richness and fertilization, we used structural equation modelling to explore possible causal relationships between trace gas fluxes, soil environmental variables (water content, NH_4^+ and NO_3^- concentrations), potential nitrifying and denitrifying activities, and gene abundances. The goal was to develop an ecologically sensible, parsimonious model for which the model-implied covariance between variables matched the observed covariance structure of the data. These covariance matrices were then compared using χ^2 tests, with significant results indicating a poor fit. Models were fitted by maximum likelihood using the lavaan software (http://lavaan.ugent.be). As with the mixed model analyses, data were first corrected for spatial gradients across the experimental site. Since not all data were present on all dates, these analyses were carried out on temporal averages of all available measurements, the underlying assumption being that effects and correlations were reasonably stable over time. Structural models were constructed under the premises that (1) processes upstream in terms of N transformation influence downstream processes (e.g. nitrification can influence denitrification), but not vice versa; (2) gene abundances can influence microbial community activities, but not vice versa; (3) trace gas fluxes can be influenced by all variables; and (4) the experimental treatments can directly influence all variables. In an educated stepwise process, we then searched for the most parsimonious model that explained the observed covariance structure adequately (insignificant χ^2 test; P was larger than 0.5 for all corresponding, indicating a very good fit). This was achieved by omitting paths with small, non-significant coefficients.

Results

Soil N_2O emissions

Averaged over all sampling dates, soil N_2O emissions were left unaffected by plant species richness but increased slightly with fertilizer application ($P=0.003$). When analyzing sampling dates separately, a significant decrease of N_2O emissions with diversity was found on April 21, 2008 ($P=0.02$), and significant species richness by fertilizer application interactions were found on June 20, 2007 ($P=0.01$) and March 11, 2008 ($P=0.05$), with the same effect being marginally significant on April 21, 2008 ($P=0.07$). These interactions resulted from the fact that the difference in N_2O emissions between fertilized and control subplots was larger at high than at low plant species richness.

Emissions of N_2O were strongly controlled by the presence of legumes. We therefore separately analyzed plots that were sown with and without legumes (Fig. 1). In the absence of legumes, species richness slightly reduced N_2O emissions, while N application slightly increased emissions (both effects marginally significant at $P=0.06$). In plots with legumes, N_2O emissions were significantly correlated with the fraction of legume biomass recovered in the summer harvest ($P=0.001$) and increased with fertilizer application ($P=0.02$), but were unrelated to plant species richness.

Soil CH_4 uptake

Averaged over all sampling dates, soil CH_4 uptake rates decreased with increasing plant species richness ($P=0.02$), but remained unaffected by fertilizer application (Fig. 2) and by the abundance of legumes. This effect was remarkably stable over time, with a decrease in CH_4 uptake with species richness detected on October 18, 2007 ($P=0.05$) and June 6, 2008 ($P=0.002$), a marginally significant decrease found on June 20, 2007 ($P=0.08$) and non-significant trends in the same direction on the other sampling dates.

Nitrifying and denitrifying enzyme activities and functional gene abundances

Nitrifying enzyme activity (NEA) increased with plant species richness ($P<0.05$), irrespective of fertilizer application (Table 2). In contrast, denitrifying enzyme activity (DEA) increased with fertilizer application ($P<0.001$), irrespective of plant species richness (Table 2).

Plant species richness did not affect functional gene abundances, although *nxrA* showed a marginally significant ($P=0.06$) and weak decrease with plant diversity. In contrast, the abundances of AOB, *Nitrobacter*-like NOB and *nirK*- and *nirS*-like denitrifiers increased with fertilizer application (Table 2).

Structural equation models explaining N_2O and CH_4 emissions

In the absence of sown legumes, N_2O emissions were affected negatively by plant species richness, as indicated by a strong direct negative path coefficient (Fig. 3, top). Plant species richness increased soil moisture, resulting in increased potential nitrification (NEA), which in turn stimulated DEA. However, DEA was not significantly linked to N_2O emissions. When plant communities contained legumes (Fig. 3, bottom), the percentage of legumes (measured as aboveground biomass fraction harvested in June) dominated the effects on N_2O emissions. Since the fraction of legumes in the plant community was negatively correlated with species richness and fertilizer application, these treatments thus reduced emissions by this pathway. However, plant species richness stimulated N_2O emissions via increases in soil moisture, which in turn increased NEA (and DEA, but as in the legume-free plots no significant link between DEA and N_2O fluxes was detected). Soil ammonium or nitrate concentrations had no explanatory power in the analysis and were therefore dropped from all models.

While the net effect of plant species richness on soil CH_4 oxidation was negative, path analysis suggested that a positive indirect effect of richness via increased soil moisture and increased NEA also was at play. However, this positive effect was masked by a larger direct negative effect on CH_4 uptake (Fig. 4). Fertilizer application stimulated AOB abundance, which positively correlated with nitrification activity. Legume abundance also boosted NEA. Ultimately, NEA had a positive effect on CH_4 uptake. Soil ammonium concentration had no explanatory power in this analysis

Structural equation models linking functional gene abundances with NEA and DEA

Path analysis did not reveal any significant links between potential nitrification and denitrification activities on one hand, and the abundances of AOB, *Nitrobacter*-like NOB or *nirK*-like denitrifiers on the other hand (Fig. 5). Due to limited amounts of extracted soil DNA available, *nirS* abundances could only be determined for both sampling dates in 83 plots, for one sampling date in another 63 plots, and not at all in 10 plots. Analysis of the subset for which both *nirS* and *nirK* abundances were available revealed a similar pattern, i.e. no link between *nirS* and enzyme activities (Online Resource 1).

At the level of enzyme activities, the effects of plant species richness were mediated by soil moisture, while the effects of fertilizer application were mediated by a reduced legume fraction in plant communities. At the level of microbial functional group abundances, N fertilization had a clear positive effect on the abundance of AOB, with cascading effects on the abundance of *Nitrobacter*-like NOB and *nirK*-like denitrifiers (Fig. 5). *nirS*-like denitrifier abundances were affected positively by N fertilization, both directly and indirectly via AOB and Nitrobacter-like NOB (Online Resource 1).

Discussion

Plant species richness decreased soil N_2O emissions—at least in the absence of legumes—and decreased soil CH_4 uptake. We also detected plant diversity effects on underlying soil microbial processes and the abundances of soil microbial groups related to these processes. However, structural equation modelling suggested that these different effects were linked in a complex way, with net effects on trace gas fluxes emerging from a combination of direct and indirect species richness effects; in this context, "direct" denotes effects not likely related to the dependent variables observed.

Structural equation modelling suggests that the effects on N_2O fluxes were at least in part mediated by positive effects of plant species numbers on soil moisture, which in turn led to accelerated inorganic N cycling, as evidenced by increased NEA and DEA. Positive path coefficients to N_2O fluxes (Fig. 3) suggest that this increase in N transformation capacity indeed stimulated N_2O emissions. At the same time, the structural equation models support a direct negative effect of plant species richness on N_2O emissions, which counteracted the positive effects of increased enzyme activities. A possible mechanism underlying this link may stem from disproportionate effects of species richness on soil inorganic N concentrations and the enzymatic potential (in our study: NEA and DEA) to transform this inorganic N. Many studies have shown more efficient soil inorganic N capture and thus lower soil nitrate concentrations in more species-rich plant communities (Ewel et al. 1991; Niklaus et al. 2001; Tilman et al. 1996). In the Jena experiment, Leimer et al. (2014b) found that soil nitrate concentrations were reduced at high diversity if no or only few legumes were present, which is compatible with the general trend found in other studies. In our analysis, however, soil inorganic N concentrations were unrelated to explanatory variables analyzed. One reason may be that our measurements were not frequent enough to estimate robust average concentrations of these very dynamic parameters. When legumes were present in experimental communities, their fractional contribution to plant community biomass was positively related to nitrification and soil N_2O emissions. In the structural equation models, the negative direct effect from species richness to N_2O emissions that we identified in the absence of legumes vanished (Fig. 3). This observation is compatible with the finding by Leimer et al. (2014b) that soil nitrate concentrations increased with diversity when a large fraction of species were legumes, indicating that a high legume diversity also led to high nitrate concentrations, and that this effect exceeded the higher inorganic N capture efficiency in more diverse plant communities that was otherwise found.

Net soil CH_4 uptake decreased persistently with plant species numbers. The soil's CH_4 balance is determined by the difference between methanogenesis and CH_4 oxidation, with the latter dominated by methanotrophic bacteria. We did not observe net CH_4 emissions during our study, indicating that CH_4 oxidation consistently outweighed methanogenesis; in

fact, we think that methanogenesis did not play an important role in the present system and that the observed patterns were thus solely caused by variation in methanotrophic activity. Soil CH_4 oxidation is often limited by gas transport rates, which in turn depend on water filled pore space (Ball et al. 1997). Increased soil moisture at high plant species richness could therefore explain the observed pattern. However, structural equation modelling suggested indirect positive effects of moisture on CH_4 oxidation via increased nitrification (Fig. 4). This mechanism is plausible, given that methanotrophy can be inhibited by high ammonium concentrations; under these conditions, all processes consuming soil ammonium will indirectly promote CH_4 oxidation. Apart from nitrification, ammonium assimilation by plants and immobilization in microbial biomass would exert such an effect. Path coefficients further suggested a soil moisture-independent direct negative effect of richness on soil CH_4 uptake. This effect persisted even if a (non-significant) link from soil moisture to CH_4 uptake was added to the model, suggesting that this effect was unrelated to soil moisture. The potential mechanisms supporting this link remain elusive; however, it also should be noted that path models do not provide strict tests of causalities but rather indicate possible mechanistic links based on correlations. The ecology of soil methanotrophic bacteria consuming atmospheric CH_4 is only poorly understood to date, in part due to the lack of success in isolating such organisms from soils (Dunfield 2007). However, it is known from experiments with intact soil cores and with available laboratory cultures that CH_4 oxidation is negatively affected by many chemical compounds, including ethylene (Jäckel et al. 2004), some organic acids (Wieczorek et al. 2011), and terpenes (Amaral et al. 1998); one possibility is that the detrimental effect of these substances results from the lack of specificity of methane-monooxygenase, which leads to co-metabolic activity harmful to the bacteria, e.g. by suicide activation (Mahendra and Alvarez-Cohen 2006; Prior and Dalton 1985). In this light, one possible explanation for the soil-moisture independent reduction of CH_4 uptake in plant species-rich communities could be an increased production of such compounds inhibiting CH_4 oxidation.

It is surprising that soil inorganic N concentrations were not significantly related to any other variable measured, whereas species richness, fertilization, and legume presence had significant effects on these parameters. This could be due to the fact that soils are spatially heterogeneous, and so are the biogeochemical transformations we investigated. For example, denitrification often occurs in the micro-anaerobic parts of soil aggregates (Sexstone et al. 1985). On the other hand, recent micro-autoradiographic analyses indicated that CH_4 assimilation preferentially takes place on aggregate surfaces to which CH_4 can readily diffuse (Stiehl-Braun et al. 2011a; Stiehl-Braun et al. 2011b). Relating system-level processes such as land-atmosphere trace gas fluxes to bulk soil measures of putative drivers, e.g. to soil inorganic N concentrations or enzymatic activities, neglects the micro-scale character of the interactions involved. When processes respond non-linearly to substrate

availability, different effects will be found when a resource such as nitrate is homogeneously or heterogeneously distributed. Our measurements, as is typical for ecosystem-level field studies, integrate the small-scale heterogeneity and thus are unable to account for small-scale interactions in hot spots and at hot moments, which show episodic disproportionately high biogeochemical transformation rates (McClain et al. 2003). We consider it likely that the marked effect of legumes is related to such heterogeneity. For example, high local concentrations of nitrate can occur in the vicinity of legume nodules, possibly creating hot-spots of denitrification and N_2O emissions. Efficient capture of inorganic N by neighboring plants will not draw down inorganic N concentrations at these locations, although it will reduce nitrate leaching losses. Such localized high concentrations will be difficult to detect in bulk soil measurements. This reasoning is in-line with a previous investigation in which we found nitrification to be only weakly related to ammonium and denitrification only weakly related to nitrate, whereas legume presence had comparably large effects (Le Roux et al. 2013). With respect to CH_4 fluxes, high bulk soil ammonium concentrations may not affect CH_4 oxidation if they occur in soil space that is distinct from the spatial niche of the active methanotrophs (Hartmann et al. 2011; Stiehl-Braun et al. 2011a).

The quantification of microbial guilds by functional gene abundance promised some insight into the community dynamics underlying N transformations. In our study, different experimental treatments were reflected differently in functional gene abundances and in enzyme activities, and no links between the two could be detected in structural equation models. Soil moisture-mediated effects of plant species could be detected in enzyme activities but not in the community size of the respective microbial guilds. On the other hand, fertilizer effects exerted a cascading effect via increased ammonia oxidizer communities to nitrite oxidizers and denitrifiers. Interestingly, no link from nitrite oxidizer gene abundances to *nirK* gene abundances was detected (Fig. 5), although the latter is downstream of the first in terms of N transformation processes. This relationship can be understood in the light of ammonium (and not nitrite) oxidation being the rate-limiting step of nitrification. Probably reflecting differences in ecology of denitrifier groups (Xie et al. 2014), *nirS* gene abundances were dependent on ammonia oxidizer 16S genes abundances indirectly via nitrite oxidizer genes. Overall, however, ammonia oxidizer community size appeared to control denitrifier community size, mostly directly but to some extent also indirectly via intermediate (in terms of N processing) nitrite oxidizer communities. Environmental controls rather than community size have also been detected in contexts other than species richness (e.g. fertilizer and drought effects: Hartmann et al. 2013; land use change: Attard et al. 2011), underlining that gene abundance data reflect biological activities only under specific conditions.

Our study revealed that many effects of plant species richness on microbial processes were mediated by effects on soil moisture. Reports on species richness effects on soil

436 moisture are equivocal (Caldeira et al. 2001; Rosenkranz et al. 2012; Spehn et al. 2000);
437 Caldeira et al. (2001) reported positive effects of species richness on grassland top soil
438 moisture but negative effects below 15 cm depth. In the Jena experiment, Rosenkranz et al.
439 (2012) found similar positive effects on top soil moisture as we did, while Leimer et al.
440 (2014a) detected no such effects. In general, patterns in soil moisture seem to have been
441 caused by decreased evaporative losses under higher species richness due to soil surface
442 shading, with concomitant increase in water capture from deeper soil layers due to better
443 soil exploration by roots. If processes are soil moisture driven, the depth at which the
444 respective microbial transformations take place therefore is critical. N_2O production due to
445 nitrification and denitrification often occur in the top soil; soil CH_4 oxidation generally also
446 dominates in the top soil, whereas methanogenesis often occurs deeper in the soil (Conrad
447 1996).

448 The patterns that emerged from our study, in particular from structural equation
449 modelling, were robust and coherent, integrating well into our current understanding of the
450 fundamental ecological processes. With respect to the fluxes of N_2O and CH_4 per se, one
451 must note that the effects of species richness and fertilization were small compared to the
452 total variation in our data, which commands a cautious interpretation (Low-Décarie et al.
453 2014). One reason for the large fraction of unexplained variance may be that the between-
454 plant community variation is typically very high in experimental plant diversity
455 experiments, in particular at low species richness. The observed diversity effects thus result
456 from averaging over the large inter-community variation within plant species richness
457 levels. In the Jena experiment, as in many field biodiversity studies, specific species
458 combinations are not replicated in independent plots, so that the diversity-independent
459 composition effect cannot be separated from unrelated (random) variation, with the
460 exception of simple contrasts such as the presence or absence of specific plant functional
461 types. When studying trace gas dynamics and the underlying microbial processes, one
462 further is confronted with large spatial and temporal variation inherent to the processes.
463 Despite this challenging variability, the present field study is the first to present clear and
464 consistent responses of soil trace gas exchange to plant species richness and fertilization.
465 Controlled experimental studies as the present one complement observational research
466 carried out under more natural conditions but in which it is more difficult to isolate effects
467 of plant diversity from potentially confounding factors (e.g. Keil et al. 2015; Sutton-Grier et
468 al. 2011).

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623 # Tables

624 Table 1. Environmental and meteorological conditions during the N_2O and CH_4 flux
625 measurements. Soil temperature and moisture refer to 8 cm depth, and are daily averages.
626 Note that the data for June 8, 2008 refers to one day-time and one night-time measurements.

627

Date	Soil temperature	Soil moisture	Precipitation	
			past 24h	past week
	(°C)	(m^3 H_2O m^{-3})	(mm)	(mm)
20. June 2007	21.6	28.0	0.0	1.0
18. Oct 2007	10.1	30.5	0.0	0.0
11. March 2008	6.8	36.7	0.0	0.1
21. April 2008	8.9	35.9	0.0	0.1
6. June 2008	20.6	23.2	0.0	0.7

628

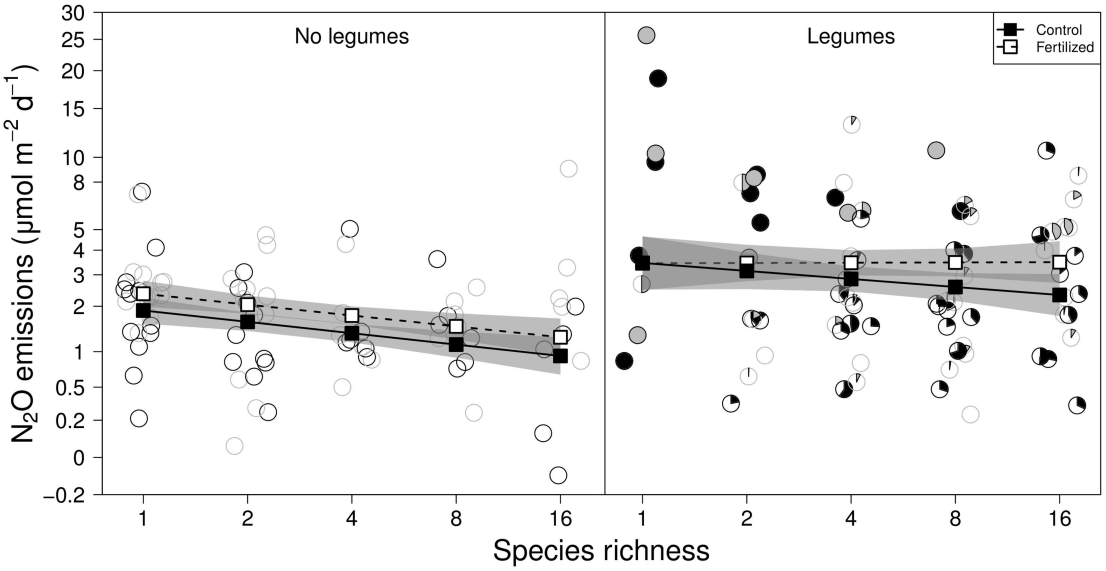
629

630 Table 2. Effects of plant species richness (logSR: log-linear effect) and fertilizer application
631 (Fert) on nitrifying and denitrifying enzyme activities (NEA and DEA) and the abundances
632 of ammonia oxidizing bacteria (AOB), *Nitrobacter*-like nitrite oxidizing bacteria (NOB), and
633 *nirK*-and *nirS*-like denitrifiers (*nirK* and *nirS*). Effect sizes are given for model-predicted
634 changes along the full plant species gradient of 1 to 16 species. Gene abundances are
635 modelled on a log scale, and effect sizes thus are given from the back-transformed model
636 prediction.

Parameter	Significance of effects			Effect sizes	
	logSR	Fert	logSR × Fert	logSR	Fert
NEA	*	n.s.	n.s.	+10%	
DEA	n.s.	***	n.s.		+7%
AOB	n.s.	***	n.s.		+51%
NOB	(*)	**	n.s.	(-27%)	+47%
nirK	n.s.	*	n.s.		+11%
nirS	n.s.	***	n.s.		+138%

637

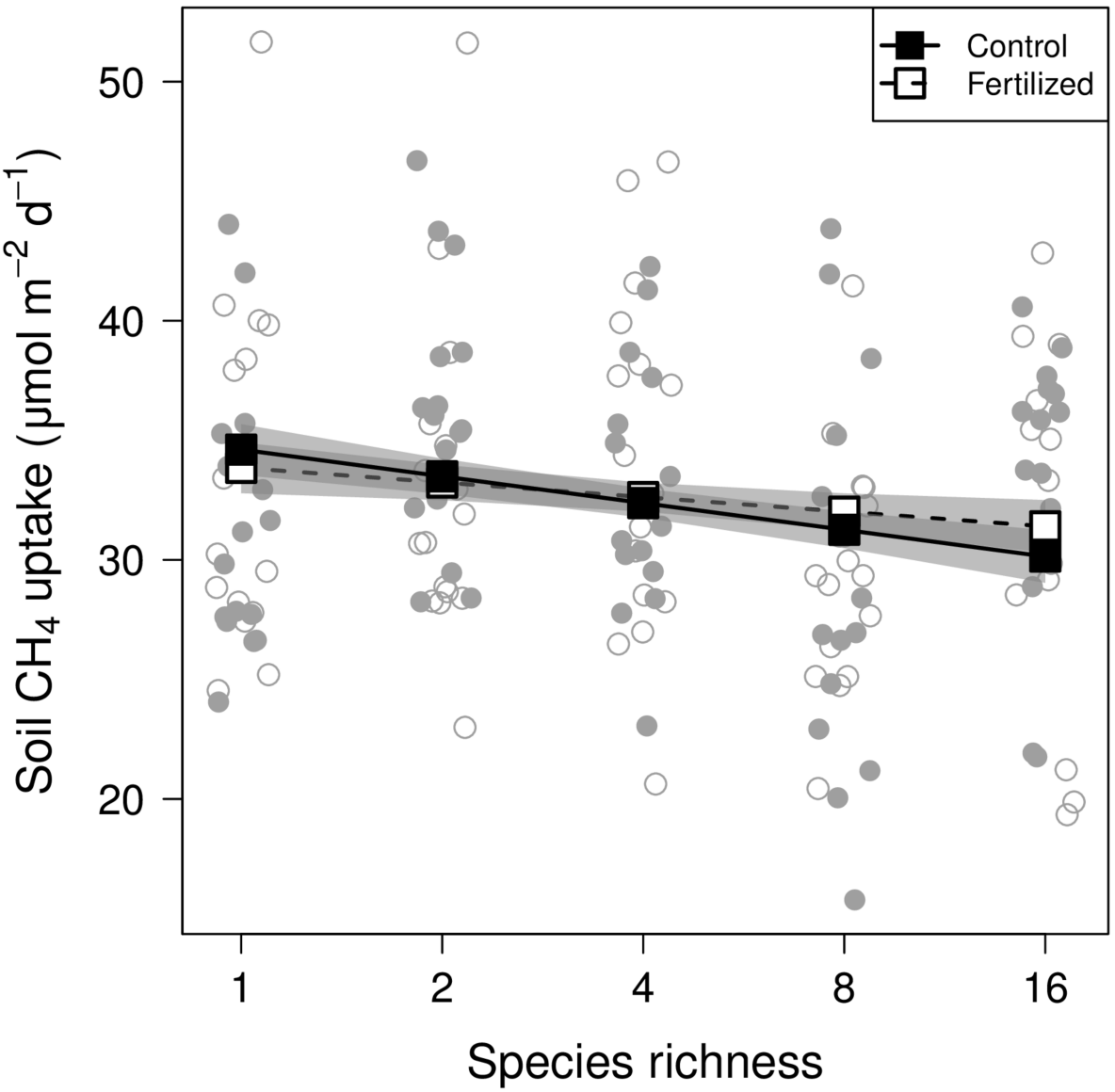
638 # Figures



639

640 Fig. 1. Soil N_2O emission rates for plots without (left) and with (right) legumes planted.
641 Individual symbols show values for each plot (dark = control; light = fertilized plots), with
642 pies inside symbols indicating the fraction of legumes in aboveground plant biomass
643 harvested in June 2007 and 2008. Square symbols and lines indicate model predicted means
644 for both fertilizer treatments; shaded areas are corresponding standard errors.

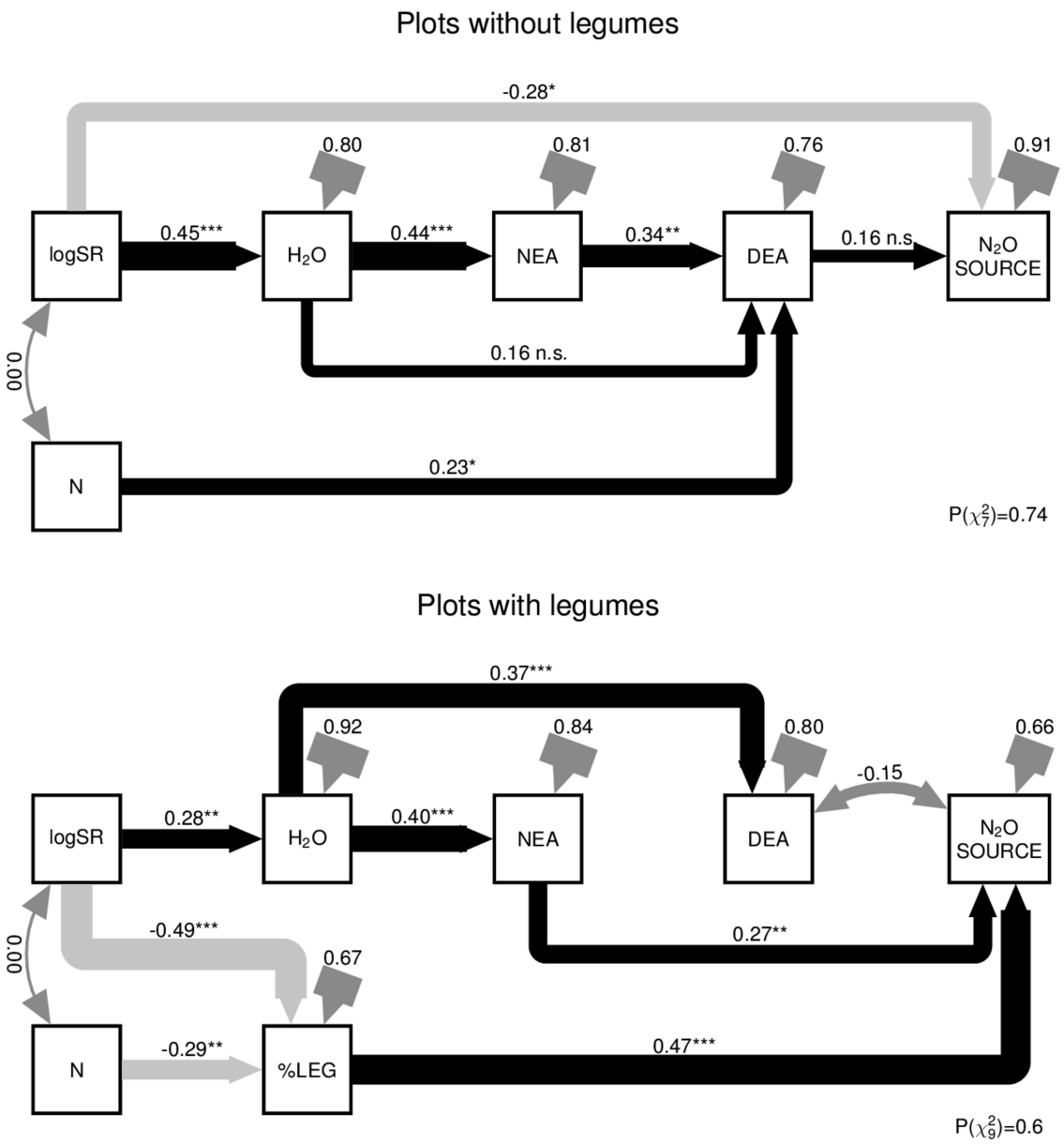
645



646

647 Fig. 2. Net soil CH_4 uptake rate as function of plant species richness and fertilizer
648 application. Closed and open circles indicate values for each control and fertilized subplot,
649 respectively. Squares and lines indicate model-predicted means for both fertilizer
650 treatments; shaded areas are corresponding standard errors.

651



652

653 Fig. 3: Path diagram with standardized path coefficients showing effects of plant species
654 richness (log-transformed, "logSR") and fertilizer ("N") on soil moisture ("H_2O"), soil
655 microbial nitrification and denitrification enzyme activities ("NEA" and "DEA"), and soil
656 N_2O emissions ("N_2O SOURCE"), separately for plots without (top) and with (bottom)
657 legumes. Arrows indicate standardized path coefficients (light: negative; dark: positive)
658 together with their significance (* P<0.05, ** P<0.01, *** P<0.001). "%LEG" indicates the mean
659 fraction of legume aboveground biomass harvested in June 2007 and 2008. χ^2 statistics
660 indicate no significant deviation of measured and model-implied covariance structure.

661

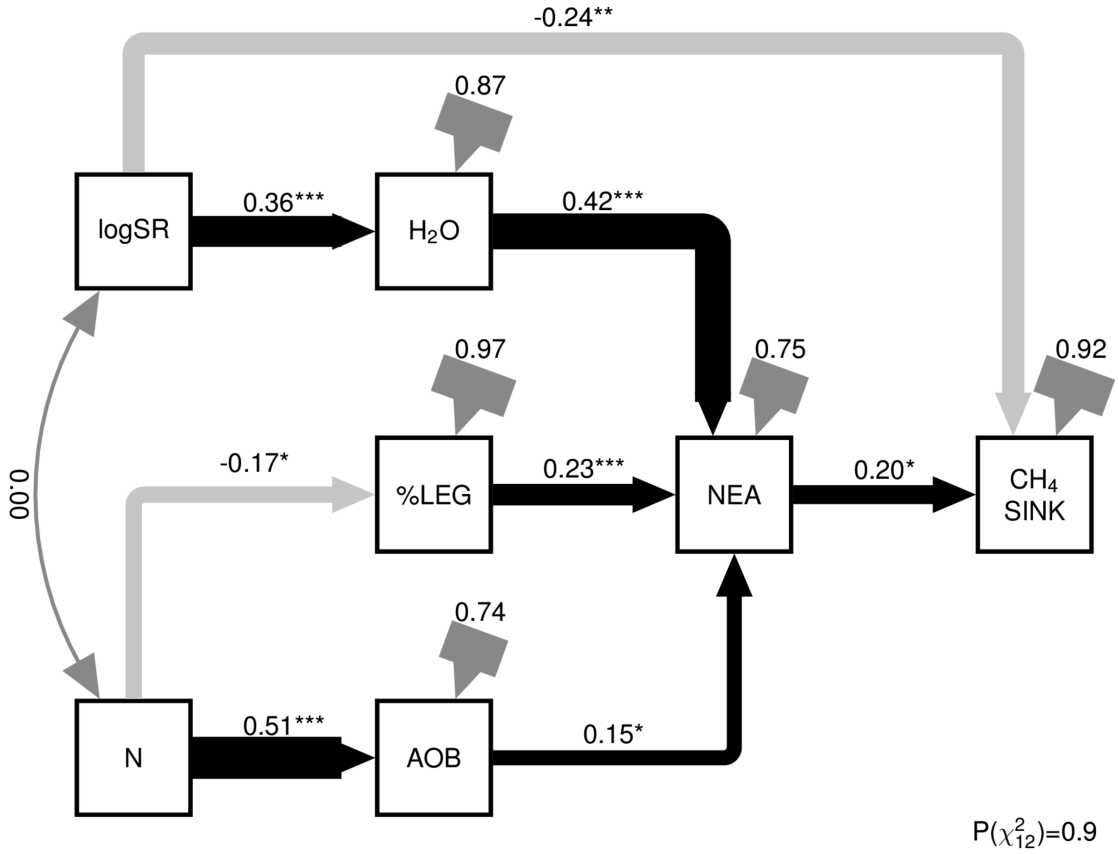
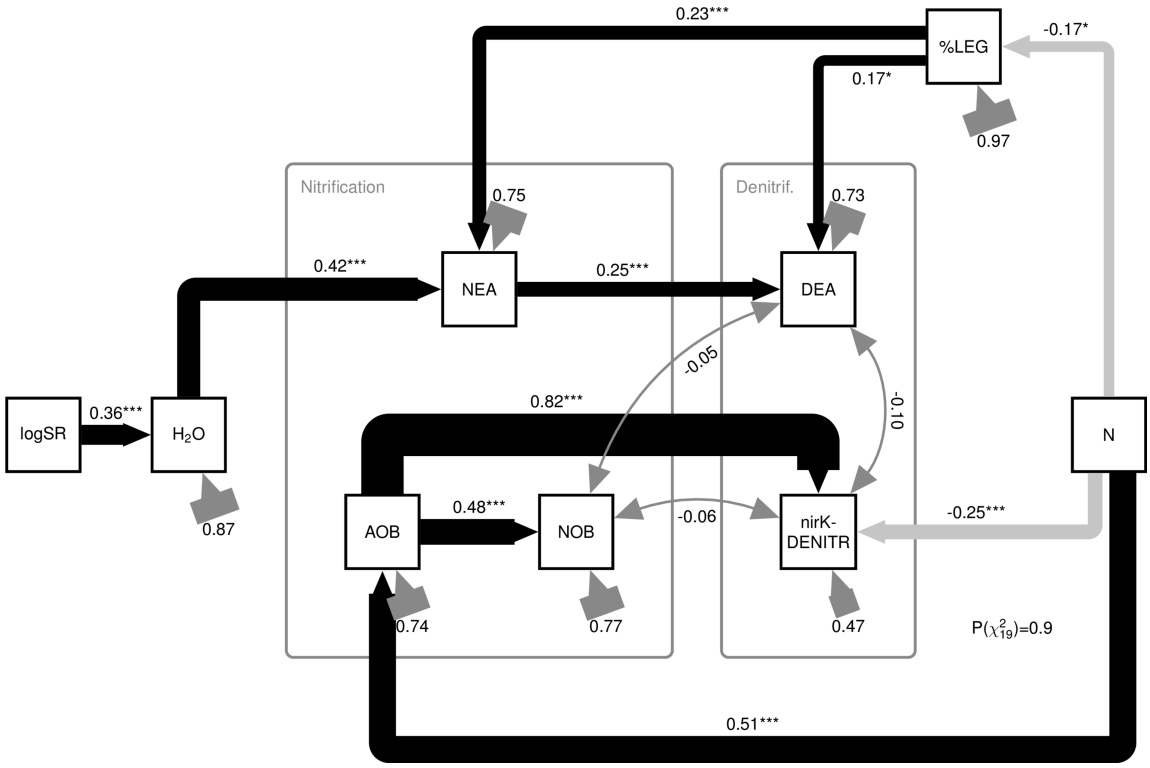


Fig. 4. Path diagram showing effects of plant species richness ("logSR") and fertilizer application ("N") on soil moisture ("H_2O"), nitrification enzyme activity ("NEA"), the abundance of ammonia oxidizing bacteria ("AOB") and soil CH_4 uptake ("CH_4 SINK"). Arrows indicate standardized path coefficients (light: negative; dark: positive) together with their significance (* P<0.05, ** P<0.01, *** P<0.001). "%LEG" indicates the mean fraction of legume aboveground biomass harvested in June 2007 and 2008. χ^2 statistics indicate no significant deviation of measured and model-implied covariance structure.



671

672 Fig. 5. Path diagram showing relationships between species richness ("logSR"), fertilizer
673 application ("N"), nitrifying and denitrifying enzyme activities ("NEA" and "DEA"), and
674 abundances of key microbial functional groups ("AOB": ammonia oxidizing bacteria,
675 "NOB": *Nitrobacter*-like nitrite oxidisers, "nirK-DENITR": *nirK*-like denitrifiers). Arrows
676 show standardized path coefficients (light: negative; dark: positive) together with their
677 significance (* P<0.05, ** P<0.01, *** P<0.001). The correlation between the exogenous
678 variables "logSR" and "N" is zero and omitted for clarity. χ^2 statistics indicate no significant
679 deviation of measured and model-implied covariance structure.